The class V myosin motor, myosin 5c, localizes to mature secretory vesicles and facilitates exocytosis in lacrimal acini

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Marchelletta RR, Jacobs DT, Schechter JE, Cheney RE, Hamm-Alvarez SF. The class V myosin motor, myosin 5c, localizes to mature secretory vesicles and facilitates exocytosis in lacrimal acini. Am J Physiol Cell Physiol 295: C13–C28, 2008. First published April 23, 2008; doi:10.1152/ajpcell.00330.2007.—We investigated the role of the actin-based myosin motor, myosin 5c (Myo5c) in vesicle transport in exocrine secretion. Lacrimal gland acinar cells (LGAC) are the major source for the regulated secretion of proteins from the lacrimal gland into the tear film. Confocal fluorescence and immunogold electron microscopy revealed that Myo5c was associated with secretory vesicles in primary rabbit LGAC. Upon stimulation of secretion with the muscarinic agonist, carbachol, Myo5c was also detected in association with actin-coated fusion intermediates. Adenovirus-mediated expression of green fluorescent protein (GFP) fused to the tail domain of Myo5c (Ad-GFP-Myo5c-tail) showed that this protein was localized to secretory vesicles. Furthermore, its expression induced a significant (P < 0.05) decrease in carbachol-stimulated release of two secretory vesicle content markers, secretory component and syncollin-GFP. Adenovirus-mediated expression of GFP appended to the full-length Myo5c (Ad-GFP-Myo5c-full) was used in parallel with adenovirus-mediated expression of GFP-Myo5c-tail in LGAC to compare various parameters of secretory vesicles labeled with either GFP-labeled protein in resting and stimulated LGAC. These studies revealed that the carbachol-stimulated increase in secretory vesicle diameter associated with compound fusion of secretory vesicles that was also exhibited by vesicles labeled with GFP-Myo5c-full was impaired in vesicles labeled with GFP-Myo5c-tail. A significant decrease in GFP labeling of actin-coated fusion intermediates was also seen in carbachol-stimulated LGAC transduced with GFP-Myo5c-tail relative to LGAC transduced with GFP-Myo5c-full. These results suggest that Myo5c participates in apical exocytosis of secretory vesicles.

actin; lacrimal gland; tear film

THE LACRIMAL GLAND (LG) is the principal source of proteins released into the tear film. These proteins play critical roles in protection of the ocular surface from pathogens and also provide nutrients and growth factors essential for maintenance of the cornea (3, 50). The lacrimal gland acinar cells (LGAC) constitute ~85% of the LG and are largely responsible for this regulated secretion of tear proteins including secretory immunoglobulin A (sIgA), secretory component (SC), lysosomal hydrolases, and growth factors, among others (50). The development of decreased LG output occurs in individuals with syndromes ranging in severity from mild dry eye to the autoimmune disorder, Sjögren’s syndrome (SjS) (12, 50). In the most severe case, SjS, initial changes in the LG are followed by lymphocytic infiltration of the gland, resulting in functional atrophy and some destruction of the tissue (12). However, LG biopsies have shown that the actual destruction of the tissue in patients with SjS is insufficient to account for the extreme decrease in LG output (12), raising the possibility that the functional atrophy of the LG may be reversed to provide some relief for affected patients. Such a strategy would depend on a mechanistic understanding of the normal effectors that regulate secretion in the gland.

We and others have established that mature secretory vesicles (mSVs) sized ~0.5–1 μm are located beneath the actin-enriched apical plasma membrane (APM) domain of LGAC (14). These SVs are enriched in the small GTPase, Rab3D (47). Recent preliminary data in our laboratory and studies in acinar epithelial cells from pancreas and parotid gland (13, 49) suggest that mSVs may also be enriched in Rab27a and/or Rab27b. Exposure of the LG to neurotransmitters released from innervating parasympathetic and sympathetic neurons triggers exocytosis of mSV at the APM and can be mimicked in vitro by agents such as the muscarinic agonist, carbachol (CCh). Apical exocytosis is accompanied by significant actin filament remodeling including thinning of the apical actin layer (cortical actin/terminal web) separating mSVs from the APM (14). In parallel, assembly of an actin coat around the base of multiple fusing mSVs and contraction of this network occurs; we have proposed that this formation of an actin-coated fusion intermediate is important in facilitating compound fusion and extrusion of the contents of these vesicles (14). Nonmuscle myosin II has been implicated in contraction of the actin coat around fusing vesicles in LGAC, but inhibition of its activity only partially impairs CCh-stimulated exocytosis and actin remodeling, suggesting that additional actin-dependent motors may participate in these events.

Myosins are a superfamily of proteins that consist of a conserved NH2-terminal motor domain (head) that associates with actin filaments and can generate force, a neck region that binds light chains, and a class-specific COOH-terminal tail (1, 18, 27, 29, 32). One of the most ancient classes of myosins are the class V myosins (11, 33). Class V myosins are expressed in organisms as diverse as fungi, Saccharomyces cerevisiae, Drosofila melanogaster, and mammals and are leading candidates to function as motors for actin-based organelle transport.

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bodies were purchased from Rockland (Gilbertsville, PA) for use. Conjugated goat anti-rabbit and goat anti-mouse secondary antibodies were used. Polarized secretion can also be blocked by treating wild-type yeast with latrunculin A; this and other evidence indicates that Myo2p binds to secretory vesicles and transports them along actin cables to sites of polarized secretion (17, 37).

Vertebrates express three class V myosins: myosin 5a (Myo5a), myosin 5b (Myo5b), and myosin 5c (Myo5c) (32, 35). Loss-of-function Myo5a leads to a defect in subcellular localization of melanosomes (pigment granules) (51). Myo5b, the second of the class V myosins to be discovered in vertebrates, is associated with a plasma membrane recycling compartment in several cell types (10, 20, 22, 24, 38, 46, 54).

Myo5c, the third member of the class V myosin family in vertebrates, is expressed most abundantly in exocrine secretory tissues. Myo5c localizes to the apical domain of epithelial cells and is hypothesized to function as a motor for actin-based organelle trafficking (35). In HeLa cells, the expression of a DN Myo5c tail led to an accumulation of transferrin receptors in large cytoplasmic puncta and inhibited transferrin recycling (35). Interestingly, Myo5c, as well as several Rab proteins, were identified in a proteomics study as components on secretory granules of pancreatic acinar cells (6). The distribution and abundance of Myo5c in exocrine tissues prompted us to explore the function of this motor in secretory vesicle exocytosis in our LGAC model system.

MATERIALS AND METHODS

Reagents. Carbachol (CCh) was purchased from Sigma-Aldrich (St. Louis, MO). Peptatin A, tosyl phenylalanyl chloromethyl ketone, leupeptin, tosyl lysyl chloromethyl ketone, soybean trypsin inhibitor, and phenylmethane sulphanyl fluoride were also purchased from Sigma-Aldrich and were used in the protease inhibitor cocktail for preparation of gland or cellular homogenate as described previously (45). The antibodies to Myo5a, Myo5b, and Myo5c used in the present study have been characterized previously (35). Mouse monoclonal anti-hemagglutinin epitope (HA) antibody was purchased from Covance (Berkeley, CA). FITC-conjugated goat anti-rabbit secondary antibody, Alexa Fluor-680-conjugated donkey anti-sheep secondary antibody, Alexa Fluor-568-conjugated goat anti-mouse secondary antibody, Alexa Fluor-488-conjugated goat anti-rat secondary antibody, Alexa Fluor 647-phalloidin, rhodamine phalloidin, and Prolong anti-fade medium were purchased from Molecular Probes/Invitrogen (Carlsbad, CA). FITC-conjugated goat anti-rabbit secondary antibody was purchased from MP Biomedical (Solon, OH). Rabbit anti-green fluorescent protein (GFP) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Sheep polyclonal antiserum to rabbit polymeric immunoglobulin A receptor (pIgR) was prepared by Caprilogics (Hardwick, MA) using secretory component (SC) from rabbit gall bladder bile as antigen. Bovine serum albumin fraction (BSA) V was purchased from EMD chemicals (Gibbstown, NJ). Anti-Rab3D polyclonal antibodies were generated in rabbits against recombinant (His)_6 epitope-tagged wild-type Rab3D expressed in Escherichia coli and purified by chromatography over protein A/G agarose (Antibodies, Davis, CA) in accordance with previous studies (9). IR800-conjugated and IR700-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from Rockland (Gilbertsville, PA) for use in Western blot analysis. Blocking buffer was purchased from Li-Cor Biosciences (Lincoln, NB). Doxycycline was obtained from Clontech (Mountain View, CA).

Primary rabbit LGAC culture. Primary LGAC were isolated as described previously (14, 15, 47) from New Zealand White rabbits (1.8–2.2 kg) obtained from Irish Farms (Norco, CA) and were euthanized in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996). Experimental protocols were approved by the University of Southern California Institutional Animal Care and Use Committee. Isolated LGAC were cultured for 2–3 days in 100-mm round culture dishes at a density of 3.0 × 10^7 cells or on glass coverslips in 12-well dishes coated with Matrigel (Invitrogen) at a density of 2.0 × 10^7 cells. We have established that under these conditions, the cultured cells reestablish distinct apical and basolateral domains, form mSVs, and position these vesicles beneath lumena formed between adjacent APM of adjoining cells (7, 14, 15, 47).

Confocal fluorescence microscopy. LGAC were cultured on Matrigel-coated coverslips for 2–3 days and were then exposed without or with CCh (100 μM, 5–15 min). Cells were fixed and permeabilized with ice-cold ethanol for 10 min in –20°C as previously described (8). After fixation and permeabilization at room temperature (RT), the cells were washed three times in PBS for 5 min at RT. The cells were then blocked with 1% BSA for 15 min at RT and processed for immunofluorescence detection for the proteins of interest (Rab3D, Myo5c, pIgR, syncollin-GFP) with appropriate primary and fluorophore-conjugated secondary antibody before fixation with Prolong anti-fade mounting medium as previously described (8, 14, 15). Fixed samples were imaged using a Zeiss LSM 510 Meta NLO confocal imaging system equipped with software for quantitation of fluorescent pixel colocalization and for measurement of vesicle diameter. Use of the LSM colocalization tool with auto-threshold to assess pixel colocalization was done in accord with previous studies (15). Images obtained were then compiled in Photoshop (version 8.0, Adobe; Mountain View, CA).

For dual labeling with rabbit polyclonal antibodies against Myo5c and Rab3D, shown as Supplemental Fig. 1 (the online version of this article contains supplemental data), cells were fixed and processed as described above up to the addition of the first primary antibody. Rabbit polyclonal antibody to Rab3D was incubated with the cells for 1 h at 37°C, washed well in PBS, and then incubated with goat anti-rabbit secondary antibody conjugated to rhodamine for 1 h at 37°C. Cells were then washed well with PBS and incubated overnight in 10% BSA containing 0.4 μg/μl of whole molecule goat anti-rabbit IgG (Sigma) at 4°C. Cells were then washed well with PBS and incubated with rabbit polyclonal antibody against Myo5c for 1 h at 37°C. After washing well with PBS, cells were incubated with goat anti-rabbit secondary antibody conjugated to FITC for 1 h at 37°C. Finally, cells were washed well in PBS and mounted as described above.

Preparation and analysis of LG tissue sections. Tissue sections were processed by surgically removing the LG from an anesthetized rat and immediately immersing into 2% paraformaldehyde in PBS for 4 h at 4°C. The excised rat LG was rinsed and washed for 24 h in PBS through several changes. The rat LG was then infused with 15% sucrose over several days with three changes of the sucrose medium. The tissue was then immersed in OCT and flash frozen using dry ice and isopentane. Serial cryosections were cut at 10 μm each using a cryostat (model 5030, Bright/Hacker; Huntington, United Kingdom) and thaw-mounted onto warm slides, which were stored at 4°C until used.

Sections were encircled using a PAP pen, permeabilized using 0.5% Triton X-100 for 15 min at RT, washed three times for 15 min each, then treated with 0.05% NaBH4 for 15 min at RT. The sections were subsequently blocked with 5% heat-inactivated goat serum in PBS, incubated with rabbit anti-Myo5c antibody in 5% goat serum,
rinced three times in PBS, incubated with Alexa-488 goat anti-rabbit secondary antibody in 5% goat serum, and then rinsed three times again in PBS. During the second wash, Alexa-568 phalloidin was added to label F-actin, and slides were mounted in Prolong anti-fade according to the manufacturer’s instructions. Tissue sections were imaged with a ×63 1.4 numerical aperture lens on a Zeiss Axiovert 100-TV inverted microscope equipped with an Orca-II cooled charge-coupled device camera (Hamamatsu). Images were adjusted for optimal brightness and contrast by Metamorph Imaging software (Molecular Devices, Downingtown, PA).

Production and purification of recombinant adenovirus. An Ad-GFP-Myo5c-tail construct was generated from the human GFP-Myo5c-tail construct in pEGFP-C2 (Clontech) as described previously (35). The pEGFP-C2 Myo5c-tail was digested with NheI and XhoI restriction enzymes at the 592 bp (5’ end) and the 3919 bp (3’ end) site of the pEGFP-C2 Myo5c-tail vector, respectively. A 3327 bp fragment digest encoding GFP-Myo5c-tail was subcloned into the pShuttle vector from the Adeno X expression system kit 1 (Clontech) and was further subcloned into the Adeno X vector in accordance with the manufacturer’s protocol. A GFP-tagged Myo5c full-length construct was derived by ligating a PCR product containing the head, neck, and proximal tail into XhoI and SacI sites of the pEGFP-Myo5c tail construct. Sequence analysis verified that full-length construct in pEGFP-C2 was identical to the published sequence of human Myo5c (accession number AF272390; aa 1-1742, nt 20-5245) except for a silent T > C change at nt 907. This construct was digested with NheI and SacI [at the 592 bp (5’ end) and the 6587 bp (3’ end) of the pEGFP-Myo5c full-length vector], and the resulting fragment encoding GFP-Myo5c full length was subcloned into the pShuttle vector from the Adeno X Tet-On expression system kit 1 (Clontech) and further subcloned into the Adeno X Tet-On vector in accordance with the manufacturer’s protocol.

Ad-Rab3D-HA was kindly provided by Dr. John Williams from the University of Michigan (4). Ad-syncholin-GFP was kindly provided by Dr. Christopher Rhodes, University of Chicago (23). Ad-GFP was generated as described previously (48).

All Ad vectors were amplified in the HEK-293-derived helper cell line, QBI. Once QBI cells displayed evidence of a cytopathic effect of the transfected Ad, the virally infected QBI cells lysed with three cycles of freeze-thaw with liquid nitrogen. The supernatant was used to further infect more plated QBI cells. The process of infection and freeze/thaw was repeated until the appropriate viral titer was achieved (35). The pEGFP-Myo5c-tail was further subcloned into the Adeno X Tet-On vector in accordance with the manufacturer’s protocol.

Viral transduction. Transduction of LGAC was done in accordance with previous studies (47) on day 2 of culture. Cells were rinsed with Dulbecco’s PBS and aspirated, and medium was then replaced with fresh culture media. The LGAC were exposed to replication-deficient Ad constructs (Ad-GFP, Ad-GFP-Myo5c-tail, Ad-GFP-Myo5c-full, Ad-Rab3D-HA, Ad-syncholin-GFP) as described below, followed by aspiration of the medium, rinsing in PBS, and addition of fresh culture medium. For Ad-GFP-Myo5c-full transduction, which requires a helper virus, LGAC were incubated for 3 h at 37°C with Ad-GFP-Myo5c-full at a multiplicity of infections (MOI) of 5, rinsed once with PBS, and incubated 3 h more with the Tet-On Ad helper virus at an MOI of 5 in the presence of 1 μg/ml doxycycline. After rinsing, doxycycline was maintained in the culture medium for the duration of the experiment. All Ad constructs were incubated with LGAC at 37°C at an MOI of 5 for 1 h. After removal of virus and replacement of culture medium, LGAC were cultured another 16–18 h before analysis.

For assays analyzing release of syncollin-GFP, LGAC transduced with Ad-GFP or Ad-GFP-Myo5c-tail, both at an MOI of 1–5, were also transduced with Ad-syncholin-GFP at an MOI of 5, resulting in LGAC doubly transduced with Ad-GFP/Ad-syncholin-GFP or Ad-GFP-Myo5c-tail/Ad-syncholin-GFP. Transduction efficiencies for Ad-GFP-Myo5c-tail, Ad-GFP, and Ad-GFP-Myo5c-full plus Tet-On helper virus averaged >90%, in accord with previous studies (47). Ad-syncholin-GFP transduction efficiency was ~80%; however, because of the high efficiency of the other constructs, in dual transduction experiments, essentially all LGAC expressing syncollin-GFP also expressed GFP or GFP-Myo5c-tail.

Analysis of Myo5c-enriched vesicle diameter. LGAC were transduced with Ad encoding GFP-Myo5c-tail or GFP-Myo5c-full and Tet-On helper virus as previously described and were fixed and processed for confocal fluorescence microscopy. Transduced GFP-Myo5c-tail or GFP-Myo5c-full-expressing cells were blocked with 1% BSA and incubated with rhodamine phalloidin to label F-actin before mounting and analysis by confocal fluorescence microscopy. Only clearly defined vesicles enriched in either GFP-Myo5c-tail or GFP-Myo5c-full were evaluated with the measurement tool function using the Zeiss LSM 510 software. Vesicles were measured at their greatest diameter. Between 12–30 fields were evaluated for each condition, with 5–10 vesicles per field measured from n = 8 separate experiments of Ad-GFP-Myo5c-tail-transduced and Ad-GFP-Myo5c-full-transduced LGAC.

SDS-PAGE and Western blot analysis. LG homogenate was prepared by homogenizing one LG (0.39 gm) with three 30-s pulses on ice in 2 ml RIPA buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) containing protease inhibitor cocktail (1 mg/ml aprotinin, and 5 μg/ml leupeptin) using a PT-MR-2100 Polytron tissue homogenizer. Blots of LG lysate were probed with appropriate primary and horseradish peroxidase-conjugated donkey anti-rabbit secondary antibodies (Jackson ImmunoResearch). Immunooblots of LG lysate were developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL), and films were scanned and imaged using Adobe Photoshop. LGAC lysate was prepared by 10 passes of 3.0 × 105 LGAC through a 23-gauge needle three times on ice in 150 μl of RIPA buffer containing protease inhibitor cocktail. LGAC lysate was resolved by SDS-PAGE and analyzed by Western blotting with appropriate primary and IR-conjugated secondary antibodies, and analyzed using the Odyssey infrared imaging system (Li-Cor). Immunoreactive bands were quantified with the Odyssey imaging software (version 2.1, Li-Cor).

Transmission electron microscopy. LGAC were isolated and cultured as described above in 150-mm Petri dishes before analysis, with and without transduction with Ad constructs before processing for electron microscopy (EM) analysis. Under resting conditions and after CCh stimulation (100 μM, 5–15 min), samples were pelleted and resuspended in buffered 4% paraformaldehyde and 0.5% glutaraldehyde fixative for 2 h. After fixation, all cells were pelleted and dehydrated with a graded ethanol series before being embedded in LR white resin (London Resin). The resulting blocks were sectioned on nickel grids, glycine-reduced, blocked with donkey gold conjugate blocking solution (EMS, Hatfield, PA) and exposed to rabbit anti-human Myo5c antibody followed by 10 nm gold-conjugated donkey anti-rabbit secondary antibody (EMS). In Fig. 1D, LGAC were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer or Sorensen’s phosphate buffer (0.75 M) for 2 h then postfixed with 1% osmium tetroxide/0.8% potassium ferricyanide (EMS) in Sorensen’s phosphate buffer (0.375 M) for 2 h at RT. Where used, counterstaining was done with Sato’s lead stain and 2% uranyl acetate.

Secretion assays. Bulk protein release was measured in accordance with previous studies (14, 15). Measurement of Sc and syncollin-GFP release from LGAC grown in 12-well plates was by collection of the culture medium bathing the cells under each condition (resting or CCh-stimulated) as previously described (14, 15). The collected culture medium was then concentrated using YM-10 Microcon centrifugal filters (Millipore, Bedford, MA), samples were resolved by 12% SDS-PAGE, transferred to nitrocellulose membranes (Whatman, Dassel, Germany), and probed with the appropriate primary and IR-conjugated secondary antibodies. Band intensity from the resulting blot was quantified for resting and CCh-stimulated samples under...
Each condition using the Odyssey infrared imaging system and the Odyssey imaging software version 2.1. Band intensity was normalized to LGAC protein content per well as determined by protein assay using the micro-BCA kit (Pierce). The resulting values were expressed as a percentage of release from unstimulated acini either in the absence of transduction (for SC release) or in acini cotransduced with syncollin-GFP and GFP (for syncollin-GFP release).

Statistical analysis. For secretion assays (Figs. 4, 5, and 7 and Table 1), paired comparisons of differences in vesicle diameter (Fig. 10C) or paired comparisons of the percentage of actin-coated vesicles with GFP label (Fig. 10D), sample sets were compared using Student’s paired two sample t-test for the means for each study with a criterion for significance of $P \leq 0.05$. For comparison of vesicle diameters (Fig. 10B) under each experimental condition, assay means were compared using a one-way ANOVA followed by postanalysis using Tukey’s test. The criterion for significance was $P \leq 0.05$.

**RESULTS**

*Myo5c is enriched on large subapical vesicles in LGAC.* LGAC are grouped into functional units called acini. Within each acini, each individual acinar cell is organized around a central lumen bounded by apical plasma membrane (APM) underlaid with actin filaments (Fig. 1A). Release of tear proteins is facilitated by exocytosis of mature secretory vesicles (mSVs) at these apical domains. Contents released into lumina drain into ducts, and these small ducts ultimately converge on the main excretory duct exiting the LG for release onto the ocular surface. As shown in Fig. 1A, when Myo5c immunofluorescence was examined in frozen sections from rat LG, it is clear that the immunofluorescence associated with this protein was concentrated beneath the APM surrounding the lumena. The immunofluorescence also appeared to be associated around the periphery of large apparent vesicles (~0.5–1.0 μm), consistent with a possible association of Myo5c with mSVs. Figure 1A also shows Myo5c immunofluorescence in primary cultures of rabbit LGAC that have been grown under conditions that allow reassociation of the isolated cells into acinar-like structures (see schematic for organization of apical and basolateral domains within the reconstituted acini). In these reconstituted acini, Myo5c immunofluorescence is also very clearly concentrated immediately beneath lumena that reform between the adjacent epithelial cells and that are delineated by the subapical actin cytoskeleton. Examination of high-magnification images of LGAC labeled to detect Myo5c suggests that not all apparent large mSVs may be enriched in this protein. This is consistent with EM analysis of SV morphology and composition, showing the presence of a heterogenous SV pool in LGAC (Fig. 1D). Note that the EM image shows that SVs occupy a significant amount of the cytoplasm, extending from the area immediately beneath the APM to the cell interior.

Western blot analysis of rabbit LG homogenate confirmed the abundant expression of Myo5c in this tissue (Fig. 1B), although Myo5a and Myo5b were also detected in LG. When extracts of LGAC homogenate were further divided into insoluble and soluble (supernatant) fractions, we found that Myo5c was largely concentrated with the insoluble fraction (e.g., membranes and cytoskeleton; Fig. 1C).

Colocalization of Myo5c immunofluorescence with that of Rab3D suggests that this motor is associated with the most apically enriched mSVs. Since the largely subapical and vesicular labeling pattern of Myo5c immunofluorescence occurred in a region enriched in mSVs in LGAC, we explored its colocalization with mSV markers. Rab3D is the best characterized marker for mSV in a variety of acinar cells including LGAC (47), pancreatic acinar cells (4), and parotid acinar cells (34). We transduced LGAC with an Ad construct encoding HA-tagged Rab3D to conduct this analysis, since the antibodies we had available to Rab3D and Myo5c were both from rabbit. As we demonstrate in Fig. 2A, Rab3D-HA in transduced LGAC has a distribution comparable to that of endogenous Rab3D in resting LGAC. Rab3D-HA also exhibits the shift
from a largely subapical localization to a less subapical and more dispersed location in response to CCh stimulation that is characteristic of the endogenous protein (47). However, in transduced LGAC, particularly after CCh stimulation, Rab3D-HA can be more readily detected throughout the cytoplasm, including the areas adjacent to the basolateral membrane. This is not a basolateral enrichment, but rather reflects the high abundance of this overexpressed protein in transduced LGAC, and its concentration in spaces void of SVs, which occupy less space near the basolateral membrane.

Comparison of the distributions of Myo5c and Rab3D in Figs. 1 and 2 suggested that Myo5c may label the most apical pool of mSVs enriched in Rab3D. As shown in Fig. 2B, high-magnification images of the luminal regions of LGAC acini showed that Rab3D-HA immunofluorescence was associated with a broad array of subapical SVs extending from the APM well into the cell interior. Myo5c immunofluorescence appeared to be consistently colocalized with the most apical pool of Rab3D-HA-enriched vesicles. This finding was verified by the comparison of the fluorescent peaks associated with each marker along the line scan. When transduced cells were stimulated with CCh, there was an apparent decrease in colocalization between Myo5c and Rab3D. This change in colocalization appeared to be largely due to diminished intensity of Rab3D fluorescence adjacent to the APM. This observation was verified by comparison of the coincident peaks of fluorescence intensity associated with each marker along the line scan. The shift away from an apical Rab3D-HA enrichment is also supported by the reduction in peak intensity for this signal adjacent to the apical actin. We calculated that the extent of total Myo5c pixels (green) that were colocalized with total Rab3D-HA pixels (red) in resting acini was 42 ± 3%; in contrast, there was a statistically significant 31% decrease in the extent of total Myo5c pixels that were colocalized with Rab3D-HA pixels to 29 ± 5% (results from n = 7 preparations). The percentage of total Rab3D-HA pixels colocalized...
with Myo5c pixels was 30 ± 2% (n = 7). This lower percentage of colocalization of the total fluorescent Rab3D-HA signal with Myo5c may partially reflect the high Rab3D-HA fluorescent signal that results from overexpression, but is also consistent with our observation that not all the Rab3D-HA-enriched mSVs were enriched in Myo5c. A small but not statistically significant reduction in total Rab3D-HA fluorescence pixels colocalized with Myo5c pixels in CCh-stimulated LGAC was seen (26 ± 4%, n = 7).

Another feature that was clearly evident in the CCh-stimulated LGAC was the formation of actin-covered structures at or adjacent to the APM. Our previous data suggest that these structures encompass fusion intermediates formed by multiple fusing mSVs; moreover, we have hypothesized that contraction of the actin coat facilitates extrusion of the contents of the vesicles at select regions within the APM (14). Although Rab3D-HA was less concentrated on these actin-coated structures, the Myo5c remained highly enriched, as verified by the coincidence of peaks associated with Myo5c and actin fluorescence along the line scan. We also noted that Myo5c distribution on vesicular structures beneath the APM was patchy, particularly in CCh-stimulated LGAC.

Although the use of Rab3D-HA to transduce LGAC suggested the association of Myo5c with subapical Rab3D-enriched vesicles, overexpression of Rab3D generated a higher background signal throughout the cytoplasm that was potentially problematic. We therefore used a sequential labeling technique using rabbit polyclonal antibodies against both Myo5c and endogenous Rab3D to verify the colocalization of these markers in the resting LGAC and the apparent increase in Myo5c with actin-coated fusion intermediates in the CCh-stimulated LGAC. As shown in Supplemental Fig. 1, this analysis was consistent with the findings in Fig. 2B.

**DN Myo5c fused to GFP is also colocalized with subapical mSVs in LGAC.** Previous work has described the generation of a DN Myo5c tail construct fused to GFP (35). The tail construct is thought to elicit its DN effect by competing with endogenous Myo5c for vesicle binding sites. To express this GFP-Myo5c-tail construct in LGAC, we cloned the construct in an Ad expression system for transduction of primary LGAC. Ad reproducibly elicits between an 80–95% transduction efficiency of LGAC, as previously reported (14, 15). When syncollin-GFP is expressed in LGAC, as shown in Fig. 4A, there is considerable Myo5c associated with these mSVs in resting LGAC as well as recruited to actin-coated fusion intermediates containing syncollin-GFP in CCh-stimulated LGAC, confirming that this marker is of relevance to the Myo5c pathway in LGAC. The more diffuse syncollin-GFP below the basolateral membrane is likely Golgi and trans-Golgi network associated, since these compartments are located beneath the nucleus and toward the basolateral membrane in LGAC.

LGAC were cotransduced with syncollin-GFP and either GFP-Myo5c-tail or GFP alone, and the effects on CCh-stimulated release of syncollin-GFP assessed. Figure 4B shows a representative Western blot, indicating that LGAC transduced with GFP-Myo5c-tail had reduced syncollin-GFP released into the culture medium following CCh stimulation. Figure 4C plots the results of multiple assays showing that the total release (resting + stimulated) of syncollin-GFP in LGAC, as well as the release attributable to CCh stimulation, were both significantly reduced by GFP-Myo5c-tail expression. A slight but statistically significant increase in basal release of syncollin-GFP was also caused by GFP-Myo5c-tail. The reason for this small basal increase is unknown but may include a general efflux of overexpressed syncollin-GFP through constitutive pathways if defects in the capacity of the regulated secretory pathway were caused by with GFP-Myo5c-tail expression. It is also possible that Myo5c may act to tether resting SVs on subapical actin and somehow clutch or brake their movement. Alternatively, this may be due to subtle functional changes in the subapical actin barrier in resting LGAC caused by the GFP-Myo5c-tail. Figure 4D shows that the decrease in CCh-stimulated syncollin-GFP release is not due to changes in syncollin-GFP expression in cells expressing GFP-Myo5c-tail.

We wanted to assess an additional content marker of mSVs and chose to evaluate the release of SC from the...
subpopulation of pIgR sequestered in mSVs. In polarized epithelial cells like MDCK cells, trafficking of pIgR has largely been elucidated in the context of its movement within the transcytotic pathway, in a ligand-free form or bound to its ligand, dimeric IgA. However, our recent work has established that this receptor is considerably enriched in mSV in LGAC (15). pIgR present in preformed vesicles is slowly cleaved to release free secretory component from the extracellular domain of this protein, which is released in a bolus following CCh-stimulated exocytosis of mSVs. This sorting occurs via a unique interaction of Rab3D with pIgR to regulate its entry into and release from mSVs (9).

Figure 5A shows the immunofluorescence signal associated with pIgR/SC in LGAC. Since the antibody is to the extracellular domain of rabbit pIgR (equivalent to SC), we cannot distinguish between the intact protein versus the cleaved SC fragment by immunofluorescence. Clearly, a considerable amount of pIgR/SC immunofluorescence was detected in very large, mSV-sized vesicles immediately beneath the APM. We have established that these structures are enriched in Rab3D (data not shown). Endogenous Myo5c is colocalized with pIgR/SC in resting as well as CCh-stimulated LGAC. In particular, in the CCh-stimulated sample, both Myo5c and pIgR/SC are detected within an actin-coated fusion intermediate. Figure 5, B and C indicates the results from a sample experiment and composite experiments, respectively. These data show that the total release of SC as well as the release attributable to CCh stimulation were both significantly reduced by the GFP-Myo5c-tail. Figure 5D shows that expression of GFP-Myo5c-tail does not affect cellular pIgR and SC expression.

As shown in Table 1, overexpression of GFP-Myo5c-tail elicited no remarkable changes in the CCh-stimulated release of bulk protein, which reflects the secretagogue-enhanced trafficking of a variety of different vesicle populations including both mSV as well as vesicles trafficking through the transcytotic pathway. Combined with the finding that Myo5c and the GFP-Myo5c-tail appeared to label only a subset of the detectable large mSV in LGAC, these data suggest that the effect of GFP-Myo5c-tail is selective for certain mSV subpopulations.
EM reveals Myo5c association with mSV and actin filaments underlying mSV. Here, we report for the first time a successful attempt to observe Myo5c localization at the EM level. The results thus far suggested that Myo5c in LGAC was associated with mSVs and that it functioned in their exocytosis. To understand further the mechanisms of its involvement, we examined the cellular localization of endogenous Myo5c in resting and CCh-stimulated LGAC using immunogold labeling and EM. Figure 6 shows gold associated with endogenous Myo5c in regions surrounding the remnants of mSVs located beneath luminal regions. When the regions are expanded (Fig. 6, A′ and A″), gold labeling is clearly localized to filament-enriched regions between the clustered mSV remnants. We note that the apparent actin filaments shown here by EM are not evident by confocal fluorescence microscopy in resting LGAC, suggesting that they are not highly abundant under these conditions and/or not readily accessible to added phalloidin. Examination of endogenous Myo5c in acini exposed to CCh revealed a more abundant filament network underlying mSVs, consistent with what we detect by confocal fluorescence microscopy as actin-coated fusion intermediates. Intriguingly, these actin filaments had clearly detectable Myo5c enrichment as evidenced by the increased abundance of gold particles associated within the filament network.

Figure 6 also shows gold labeling associated with overexpressed GFP-Myo5c-tail in transduced LGAC in the presence and absence of CCh. As is evident under each condition, a dense network of gold particles was detected in regions between adjacent mSVs. The addition of CCh did not elicit any changes in gold labeling patterns at the microscopic level, in contrast to the apparent redistribution to filament-rich regions seen in nontransduced, CCh-stimulated acini. Finally, morphogenesis of regions of cytosol lacking mSV (Fig. 6C, box 1) at the basal cytoplasm did not show any evidence of significant gold labeling, suggesting that the overexpressed GFP-Myo5c-tail was specifically targeted to areas rich in mSV. Morphology in these specimens was not optimally preserved because the samples were not subjected to osmification.
LGAC overexpressing GFP-myo5c-tail exhibit changes consistent with impaired compound fusion relative to LGAC expressing GFP-myo5c-full. Although we saw evidence by confocal fluorescence microscopy (Figs. 3–5) and EM (Fig. 6) that the relationship between actin coats assembled around putative fusion intermediates and SVs might be altered in LGAC overexpressing GFP-Myo5c-tail, it was difficult to come to firm conclusions because of some loss of morphology required to preserve antigen immunoreactivity to detect Myo5c by immunogold labeling. We therefore decided to compare SV parameters using confocal fluorescence microscopy, by labeling SVs either with GFP-Myo5c-tail or with GFP-Myo5c full-length protein, which is functionally competent. We developed an Ad construct encoding GFP-Myo5c-full in a Tet-On system, to allow regulated expression of a large protein (~230 kDa). This construct, plus the Ad-Tet-On helper virus, was used to transduce LGAC, as described in MATERIALS AND METHODS.

Table 1. Bulk protein release in LGAC transduced with GFP or GFP-Myo5c-tail

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Resting, %</th>
<th>Total, %</th>
<th>CCh-Stimulated Component, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad-GFP</td>
<td>100.00</td>
<td>381 ± 67</td>
<td>281 ± 67</td>
</tr>
<tr>
<td>Ad-GFP-Myo5c-tail</td>
<td>114 ± 7</td>
<td>361 ± 68</td>
<td>246 ± 69</td>
</tr>
</tbody>
</table>

Values are means ± SE of 10 separate preparations. Resting values reflect release into culture medium over 30 min in the absence of stimulation. Total values reflect release over 30 min in the presence of 100 μM carbachol (CCh). The CCh-stimulated component reflects the “total” minus the “resting release.” All values are normalized to protein content of the cell pellet before comparison. LGAC, lacrimal gland acinar cells; Ad-GFP-Myo5c-tail, adeno-virus-mediated expression of green fluorescent protein fused to the tail domain of myosin 5c.
With overexpressed GFP-Myo5c-full or GFP-Myo5c-tail under each condition. The diameter of mSVs enriched in GFP-Myo5c-full appears larger with CCh stimulation relative to that in resting LGAC, consistent with our working model of compound fusion prior to exocytosis. However, such an increase was not readily apparent in mSV labeled with GFP-Myo5c-tail in LGAC stimulated with CCh, relative to resting LGAC. In addition, the actin coats frequently detected around subapical mSVs enriched in GFP-Myo5c-full in CCh-stimulated LGAC were infrequently detected with vesicles labeled with GFP-Myo5c-tail in CCh-stimulated LGAC.

The trends obtained from visual examination in Fig. 9 were reinforced by the quantitative analysis in Fig. 10. Reinforcing the observations obtained from confocal and EM micrographs, we found that the percentage of actin-coated vesicles, as observed by confocal as phalloidin-positive staining distinct from the cortical actin, that were labeled with GFP in CCh-stimulated acini was significantly reduced in LGAC expressing GFP-Myo5c-tail relative to GFP-Myo5c-full (Fig. 10D).

Other aspects of SV organization, although more subtle than the obvious lack of GFP-Myo5c-tail association with actin-coated granules, provided additional insights into the role played by Myo5c in exocytosis. The plots shown in Fig. 10A depict the number of vesicles of different diameter detected in resting and CCh-stimulated acini that were enriched in either GFP-Myo5c-full or GFP-Myo5c-tail. Our previous work (14) had used EM to determine the range in diameter of individual mSVs, as well as dual and multiply fused mSVs in LGAC. In this previous study, formation of dual and multiply fused vesicles of greater diameter was stimulated by CCh, consistent with activation of compound fusion. In GFP-Myo5c-full and GFP-Myo5c-tail transduced and unstimulated LGAC, both motors were enriched on vesicle populations of diameter <1 μm. However, comparison of the histogram plots as well as the calculated mean diameter values (Fig. 10B) in unstimulated LGAC revealed that the average diameter of vesicles labeled with GFP-Myo5c-tail was significantly less than those enriched in GFP-Myo5c-full.

Consistent with previous work, the analysis of diameter in vesicles labeled with GFP-Myo5c-full in CCh-stimulated LGAC relative to these vesicles in unstimulated LGAC revealed the appearance of vesicles of larger diameter. This apparent shift was verified by an increase in the average diameter of these vesicles relative to their diameter in unstimulated LGAC (Fig. 10B). In contrast, fewer vesicles of diameter >1 μm were labeled with GFP-Myo5c-tail in CCh-stimulated LGAC (Fig. 10A). While a modest and still significant increase in diameter of GFP-Myo5c-tail-enriched vesicles was seen in CCh-treated LGAC relative to unstimulated LGAC, the diameter of the GFP-Myo5c-tail-enriched vesicles in stimulated LGAC was significantly smaller than that for GFP-Myo5c-full-enriched vesicles in stimulated LGAC (Fig. 10B). As shown in Fig. 10C, the CCh-induced increase in vesicle diameter was significantly reduced by GFP-Myo5c-tail. These data, combined with the uncoupling of GFP-Myo5c-tail from actin coated vesicles, suggest that Myo5c participates in an aspect of compound fusion involving association of primed and fusing mSVs with actin coats.
DISCUSSION

Our study is the first to test the function of Myo5c in secretion from acinar epithelial cells. Here we show that endogenous Myo5c is associated with LG mSVs. Evidence for this includes the finding that endogenous Myo5c is colocalized with Rab3D in vesicles of the very large diameter characteristic of mSVs. Rab3D has been well characterized as a constituent of mSVs in acinar cells from pancreas (4), parotid gland (26), and LG (47), as well as within the lamellar bodies of type II alveolar cells (44). In addition, Rab3D has been well characterized as a constituent of mSVs in acinar cells from pancreas (4), parotid gland (26), and LG (47), as well as within the lamellar bodies of type II alveolar cells (44). In addition, Rab3D was colocalized with both GFP-Myo5c-tail and GFP-Myo5c-full in resting, transduced LGAC. Additional evidence that Myo5c is associated with mSV was provided by the demonstration of functional inhibition of CCh-stimulated SC and syncollin-GFP release from mSV by overexpression of GFP-Myo5c-tail. Finally, immunogold and EM shows clearly that Myo5c is associated with mSVs and their underlying actin cytoskeleton, particularly in stimulated LGAC. Our findings on the association of Myo5c with exocrine mSV in LGAC are consistent with a recent report using organellar proteomics that identified Myo5c as a constituent of zymogen granules in pancreas (6).

In addition to the demonstration of Myo5c on mSV in resting LGAC, our study suggests a role for Myo5c in association of actin coats around fusing mSV during exocytosis. After stimulation of LGAC with CCh, actin-coated structures appear near the APM. These structures have previously been shown to contain several mSV enveloped with an actin coat that are in the process of undergoing compound fusion (14, 15). We have hypothesized that this step precedes the extrusion of vesicle contents from this intermediate at the APM. A role for nonmuscle myosin II in contraction of the actin coat and subsequent compound fusion and extrusion of the contents within the actin-coated structure at the APM is supported by inhibitor studies. For instance, stabilization of actin coats by inhibition of nonmuscle myosin II results in accumulation of multivesicular fusion intermediates as well as inhibition of protein secretion, suggesting that actin coat assembly proceeds compound fusion and extrusion of vesicle contents (14). In the current study, endogenous Myo5c was detected in association with actin-coated fusion intermediates in stimulated LGAC by both confocal fluorescence microscopy and immunogold and EM, suggesting...
that it may participate in an actin-dependent component of compound fusion.

When Myo5c-enriched vesicle association with actin-coated structures was examined in stimulated LGAC transduced either with Ad-GFP-Myo5c-full (green; A) or Ad-GFP-Myo5c-tail (green; B) and then labeled with antibodies and affinity label to detect endogenous pIgR/SC (purple) and actin filaments (red). Colocalization of both Myo5c constructs with pIgR/SC fluorescence is shown by arrows. Colocalization was confirmed using line scan analysis (red line overlay), and the fluorescence intensity (in arbitrary intensity units) plots for each marker are shown below the images. Areas devoid of fluorescence such as the lumena (L) and actin-coated vesicles (V) are marked on the line scan. Bars, ~5 µm.

Additional analysis revealed that mSV labeled with GFP-Myo5c-full exhibited a significant increase in mean vesicle diameter in CCh-stimulated LGAC relative to resting acini, comparable to previous studies (14). However, this increase in vesicle diameter was largely blunted in mSV labeled with GFP-Myo5c-tail in CCh-stimulated LGAC, suggesting that compound fusion was affected. In addition, the histogram plot of vesicle diameters of vesicles labeled with GFP-Myo5c-tail indicated fewer large-diameter vesicles, relative to vesicles labeled with GFP-Myo5c-full.

We suggest that Myo5c functions in pairing of primed mSV with actin coats as an initial step in the exocytotic process. EM data suggest that endogenous Myo5c is largely associated with the dense network of actin around SVs in CCh-stimulated LGAC. The distribution of Myo5c (both endogenous and GFP-Myo5c-full) in CCh-stimulated LGAC is patchy, consistent with enrichment on aggregates of actin filaments. Inhibition of Myo5c function by overexpression of the tail domain would have the consequence of inhibiting compound fusion by preventing actin coats from associating appropriately with primed mSVs. Intriguingly, recent biochemical studies of human Myo5c have suggested that it functions as a low duty ratio, nonprocessive motor protein (21, 39). Myo5a has been well characterized as a highly processive motor that undergoes multiple enzymatic cycles while attached to the actin cytoskeleton; this profile is characteristic of a vesicle motor protein. Nonprocessive behavior by Myo5c means that this motor would require concerted action by multiple units to facilitate transport. This suggests that the function of Myo5c in cells may be quite different than its processive cousin, Myo5a.

Of interest in resting LGAC, when parameters of vesicles labeled with GFP-Myo5c-tail and GFP-Myo5c-full were compared, is that the vesicles labeled with GFP-Myo5c-tail were significantly smaller in diameter by ~15%, relative to vesicles labeled with GFP-Myo5c-full. Little is known about mSV
biogenesis in LGAC, but literature on maturation of mSV from immature SV in neuroendocrine and endocrine cells suggests a model of maturation by homotypic fusion (2). If a similar model is applicable in LGAC, a smaller-diameter SV population may represent a preponderance of immature SVs. The role of Myo5c in vesicle maturation could be direct (e.g., plays a role in homotypic fusion) or indirect (e.g., plays a role in recruitment of other factors necessary for maturation).

Previous work has shown that expression of a GFP-Myo5c-tail construct in HeLa cells suggests that Myo5c participates in transferrin receptor recycling (35). This finding suggested a possible role for Myo5c in apical membrane recycling in LGAC, although it is important to note that HeLa cells are nonpolarized and do not exhibit a regulated secretory pathway.

Conceivably, Myo5c associated with mSV might be passively transported to the APM in association with exocytosing mSV, and it could then play an active role in the compensatory apical endocytosis of exocytosed mSV membrane to a recycling apical endosome. Inhibition of apical endocytosis might exert a negative feedback effect on apical exocytosis due to the distension of APM and/or the depletion of membranes available for regeneration of mSV, explaining the inhibition of SC and syncoilin-GFP release exerted by the GFP-Myo5c-tail construct.

Interestingly, there is some disagreement in the literature about the precise point in acinar exocytosis at which actin-coated structures are formed. All results obtained thus far in LGAC indicate that these coats assemble before compound fusion and content extrusion (14), but a few recent reports in pancreatic acini have suggested that the actin coat assembles on zymogen granules that have just undergone exocytosis (25, 28, 41). One explanation for the need for an actin coat just after initiation of exocytosis (e.g., formation of the fusion pore) is for stabilization of the opposing membranes and/or content extrusion of the contents trapped in the large inclusion (25). The dissection of the role of the actin coat in mSV exocytosis in exocrine tissues is complicated by the differing approaches used for analysis, as well as the existence of different modes of exocytosis utilized by these tissues; sequential (pancreatic acini) versus multivesicular (parotid acini and LGAC) (28).

However, if an actin coat forms after exocytosis is initiated in LGAC, it may in fact facilitate the rapid retrieval of mSV membrane to endosomal compartments via vesicle transport on subapical actin utilizing Myo5c. We feel that this scenario is less likely than our proposed model for Myo5c participation in compound fusion and exocytosis for several reasons. First, we saw no evidence for recovery of Myo5c with endosomal compartments in CCh-stimulated LGAC by immunogold/EM; rather, the Myo5c appeared to become increasingly enriched in the apical actin meshwork that increased adjacent to fusing membranes in CCh-stimulated LGAC by immunogold/EM; and it could then play an active role in the compensatory apical endocytosis of exocytosed mSV membrane to a recycling apical endosome (22). It is possible that this scenario is less likely than our proposed model for Myo5c participation in compound fusion and exocytosis for several reasons. First, we saw no evidence for recovery of Myo5c with endosomal compartments in CCh-stimulated LGAC by immunogold/EM; rather, the Myo5c appeared to become increasingly enriched in the apical actin meshwork that increased adjacent to fusing membranes in CCh-stimulated LGAC by immunogold/EM; and it could then play an active role in the compensatory apical endocytosis of exocytosed mSV membrane to a recycling apical endosome.
sion GFP-Myo5c-tail may not fully displace all of the endogenous Myo5c from SVs. It should also be noted that partial inhibition of secretion has been noted in situations where other essential effectors have been disrupted. Partial inhibition of secretion has been observed for 5-HT release in platelets from Rab27b knockout mice, for amylase release from mouse pancreatic acinar cells expressing DN Rab3D and Rab27b, and for /H9252-hexosaminidase release in mast cells from VAMP8 knockout mice (4, 5, 31, 40).

Our data show evidence for a strong association of Myo5c with Rab3D-enriched mSV in particular. Since some myosins and kinesins have been shown to be tethered to vesicular cargo via Rab binding (19, 27, 52, 53), we considered whether Rab3D might actually bind directly to Myo5c. Coimmunoprecipitation studies from LGAC did not reveal any evidence for a strong protein-protein association between these effectors (unpublished data). As shown here, the colocalization of these two proteins was most extensive in resting LGAC, CCh stimulation resulted in a decrease in Rab3D colocalization with Myo5c of 31%, reflecting the release of Rab3D from primed fusing mSV that occurs prior to formation of the actin coat and subsequent compound exocytosis (42, 43, 47). In contrast, the Myo5c was retained with the actin coat and mSVs in CCh-stimulated LGAC, suggesting that it does not require Rab3D to retain its association with mSV or their closely associated actin filaments.

To summarize, we have conclusively demonstrated for the first time that Myo5c is associated with mSV in LGAC, and the

Fig. 10. GFP-Myo5c-tail-enriched mSVs exhibit reduced diameter in the presence and absence of CCh and reduced association with actin-coated structures in CCh-stimulated LGAC. A: diameters of GFP-Myo5c-full and GFP-Myo5c-tail-enriched vesicles in either resting or CCh-stimulated (100 μm, 15 min) were measured using the LSM 510 confocal quantification software tool. The plots show the distribution of vesicle diameters in resting LGAC (top) and CCh-stimulated LGAC (bottom) for vesicles labeled with GFP-Myo5c-full (black) and GFP-Myo5c-tail (gray) quantified from n = 8 preparations and 12–30 fields per preparation. The total vesicles counted include GFP-Myo5c-full (resting), 983; GFP-Myo5c-full (CCh-stimulated), 704; GFP-Myo5c-tail (resting), 1,295; GFP-Myo5c-full (CCh-stimulated), 859. Vesicles >3 μm in diameter were pooled. B: vesicle diameters of all vesicles counted in A were averaged by preparation (n = 8), and changes in average values were compared for statistical significance using a one-way ANOVA and Tukey’s posttest. *Significant increase from resting in the same category; #significant decrease from GFP-Myo5c-full (resting); ##significant decrease from GFP-Myo5c-full (CCh-stimulated), P < 0.05 for all. C: CCh-induced differences in vesicle diameter by treatment. #Significance at P ≤ 0.05. D: actin-coated vesicles in stimulated LGAC that were labeled with GFP were quantified and plotted as a percentage of total actin-coated vesicles. Actin-coated vesicles were characterized as phalloidin-positive vesicular structures that were distinct from cortical actin. #Significant decrease in GFP-Myo5c-tail-labeling of actin coats (P < 0.05).
preponderance of evidence suggests that it functions in exocytosis of mSVs in this system. Our data suggests a model in which Myo5c on mSVs facilitates the pairing of primed mSVs with actin coats as the compound fusion intermediate is assembled, prior to exocytosis of mSV contents.

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